Effects of *Wolbachia* in the uzifly, *Exorista sorbillans*, a parasitoid of the silkworm, *Bombyx mori*

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Abstract

The uzifly, *Exorista sorbillans* (Diptera: Tachinidae), a parasitoid of the silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae), harbours *Wolbachia* (Rickettsia) endosymbionts. Administration of 0.05 mg/ml oxytetracycline to the adult uziflies removed *Wolbachia* endosymbionts and resulted in different reproductive disorders, such as i) reduction in fecundity of uninfected females, ii) cytoplasmic incompatibility in crosses between infected males and uninfected females, iii) sterility in the crosses between both males and females from uninfected populations, and iv) sex-ratio distortion in uninfected females irrespective of the presence of *Wolbachia* in males. However, tetracycline treatment did not have much effect on longevity of the uzifly. These results suggest that the interaction of *Wolbachia* with its uzifly host is one of mutual symbiosis as it controls the reproductive physiology of its hosts.

Cite this paper as: Puttaraju HP, Prakash BM. 2005. Effects of *Wolbachia* in the uzifly, *Exorista sorbillans*, a parasitoid of the silkworm, *Bombyx mori*. 7pp. *Journal of Insect Science* 5:30, available online: insectscience.org/5.30

Keywords: fecundity, cytoplasmic incompatibility, sterility, sex ratio, tetracycline
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Received 28 November 2004 | Accepted 15 May 2005 | Published 11 November 2005
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Introduction

Maternally transmitted bacteria of the genus *Wolbachia* form a monophyletic clade of intracellular alpha-proteobacteria and are particularly related to the Rickettsia that cause human diseases such as Rocky mountain spotted fever, typhus and Q-fever (Hackstadt, 1996). *Wolbachia* have been reported to have negative, zero (Moran et al., 1994) or positive (Wade et al., 1995) effects on host fitness and is one of the most ubiquitous endosymbionts described to date, infecting a number of invertebrate hosts including mites, crustaceans, filarial nematodes and especially insects (Bandi et al., 1998). These bacteria display the ability to manipulate the reproduction of their hosts via a diverse array of phenotypes including cytoplasmic incompatibility in a wide range of insects (Barr, 1980; O'Neill et al., 1992), parthenogenesis in hymenopteran wasps (Stouthamer et al., 1993), and feminization of genetic males in Isopods (Rousset et al., 1992) and *Ostrinia furnacalis* of Lepidoptera (Kageyama et al., 2002). It has a wide host range and multiple infection sites within the host and some strains play a role in the ageing of insects by degrading different tissues in *Drosophila simulans* (Min et al., 1997), and in the enhancement of fecundity in infected females of *Asobara tabida* (Vavre et al., 1999). The elimination of *Wolbachia* by tetracycline treatment is known to decrease host fitness in several non-specific ways, including survival, developmental success, and reproduction in filarial nematodes (Hoerauf et al., 1996; Longworthy et al., 2000), and to cause infertility in females of the species of *Aedes* (Trpis et al., 1981; Kambhapathi et al., 1993) and in the parasitic wasp, *Asobara tabida* (Dedeine et al., 2001; 2004).

The uzifly, *Exorista sorbillans*, a Dipteran endoparasite, on silkworm, *Bombyx mori* L (Lepidoptera: Bombycidae) causes severe damage to the silk industry accounting for 15 - 20% of yield loss. The presence of *Wolbachia* in this pest has been reported (Madhu et al., 2001; Puttaraju et al., 2002) from our laboratory using molecular and cytological techniques, and it was found that, the *Wolbachia* present in the uzifly are similar to those of B group *Wolbachia* found in *Drosophila melanogaster* and *Culex pipiens* (Chatterjee et al., 2003). In this paper we present the data resulting from administering tetracycline in its diet, which is known to cure *Wolbachia* infection of the uzifly (Manjunatha, 1993).

Materials and Methods

Uzifly collection, rearing and crossing experiments

About three hundred plus post-parasitic maggots of uzifly (offspring of many mothers) were collected from silkworm cocoon markets at Ramanagara and Vijayapura of Karnataka, India, soon after the emergence from the infested host silkworm cocoons. The maggots were brought to the laboratory and allowed to metamorphose into pupae and then to adults in wire mesh netted cages of 14” x14” x14” inch. In order to ensure virginity, the male and female adult flies were separated immediately after emergence based on genitalia and other morphological sex- specific characters. The uziflies were divided into five groups by random selection, each with twenty-five males and twenty-five females. One group was maintained as infected population (control) by feeding with 8% glucose in distilled water soaked in cotton balls. To standardize the concentrations of tetracycline to kill *Wolbachia*, four groups of uziflies were separately fed with different concentrations of tetracycline viz. 0.01mg/ml, 0.02 mg/ml, 0.05 mg/ml and 0.10 mg/ml tetracycline in 8% glucose, for a period of three generations and another four generation the flies were maintained without tetracycline. In the fifth generation, two males (to ensure coupling) were crossed with one female with thirty replications in each of the crosses. They were allowed to mate for a period of 24 hours to insure complete insemination. The flies were allowed to lay one to three eggs on each silkworm larval bodies and these eggs were allowed to develop as maggots inside the host and to emerge out of silkworm larvae/cocoon.

Fifteen early fifth instar silkworm larvae were placed in respective cages to allow the uziflies to oviposit on them, because these uziflies prefer to lay their eggs only on silkworm larvae (Manjunatha, 1993). The host silkworm larvae were replaced every twelve hours to allow oviposition by uziflies for a period of six days. These silkworm larvae, with one to three uzi eggs on them were reared in the laboratory by following a standard silkworm rearing technique (Krishnaswamy, 1978).

The rate of fecundity was calculated by counting the individual eggs laid on each larva after 24 hours of oviposition. The number and percentage of eggs hatch were determined by counting the number of black scars, which appeared on the body of the

silkworm larvae after hatching. The host silkworms were further reared in the laboratory until the uzi maggots emerged out from the silkworm bodies. The emerged maggots were collected and maintained in the laboratory as described above. Upon emergence, the flies were counted and recorded for male: female ratios. Apart from these, in separate multiple cages, thirty-five male and thirty-five female flies in each replicate with five replications were maintained by feeding with glucose (as the controls) and tetracycline with glucose (as the experimentals) for analysis of the effects of tetracycline on longevity of *E. sorbillans*. The resulting data were analysed using one-way ANOVA and student's t-test manually (Khan et al., 1994).

Genomic DNA Isolation from uziflies

DNA from individual uziflies was extracted following the usual stepwise methods of extraction with phenol, phenol: chloroform: isoamylalcohol, chloroform and finally precipitated with two volumes of double distilled alcohol in the presence of 3M sodium acetate (Sambrook et al., 1989). The precipitated DNA was washed in 70% alcohol, dried and dissolved in 2 ml of TE (Tris-EDTA) buffer. The DNA was subjected to RNAse-A treatments followed by further re-extraction with above steps, and finally dissolved in TE buffer. The concentration of 10-12 ng/µl was confirmed through quantification on 0.8% agarose gel using standard concentration of lambda DNA in TBE (Tris –Boric acid-EDTA) buffer for subsequent PCR analysis.

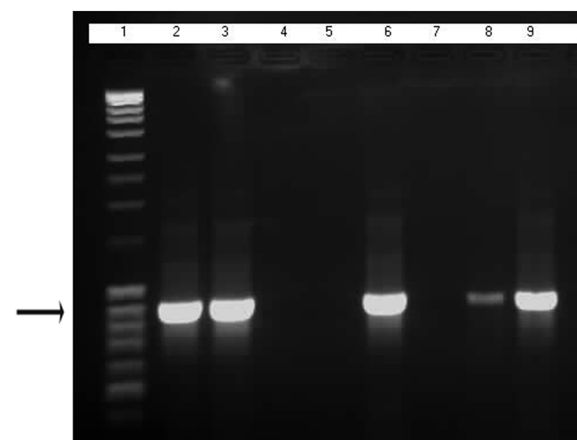
PCR-analysis

A polymerase chain reaction assay based on specific amplification of the *16S rRNA* gene primer pair 76-99F 5'- TTG TAG CCT GCT ATG GTA TAA CT - 3' and 1012-994R 5'- GAA TAG GTA TGA TTT TCA TGT - 3' which were synthesised (Bangalore Genei, www.bangaloregenei.com) based on published sequence information for the16S rRNA of *Wolbachia pipientis* (O'Neill et al., 1992) was used to detect *Wolbachia* in individual flies and it was carried out with PTC 200 of MJ Research Thermocycler, in 20 µl reaction mixture containing 1x PCR buffer, 200 µM dNTP's, 2.5 mM $MgCl_2$, and 0.5 U *Taq*-DNA Polymerase (MBI- Fermentas, www.fermentas.com), 83.33 pmole of each forward and reverse primers (Bangalore Genei), 45-50 ng template DNA and final volume of millipore water to 20 µl. The amplification was carried out with a cyclic condition of initial denaturation step at 94°C for 2 min followed by 40 cycles with denaturation step at 94°C for 1 min, primer annealing at 55°C for 2 min and primer extension with the presence of *Taq*-DNA polymerase at 72°C for 2 min in each cycle and final extension at 72°C for 5 min. The amplified PCR products were fractionated through 1.3% agarose gel electrophoresis run in 1x TBE (Tris- Boric acid- EDTA) buffer for a length of 5-6 cm at a constant volt of 65V for Pharmacia power supply EPS 200V/ 400mA. The gel was stained with 0.5 µg/ml gel of ethidium bromide just prior to casting. The documentation was done with gel documentation system with Genesnap software version 5.0. Triplicate PCR experiments were carried out on different occasions for reproducibility of expected band size.

Results

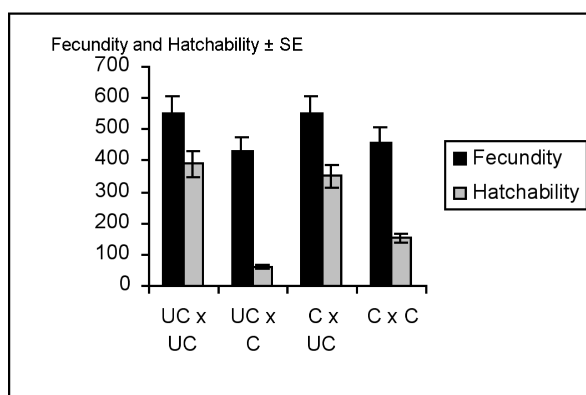
Among the different concentrations of tetracycline tested, 0.05 mg/ml and 0.10 mg/ml were found effective in curing / reducing *Wolbachia* populations which, in turn drastically affected the reproductive biology of the uzifly. Of the two concentrations, 0.05 mg/ml was used for eliminating *Wolbachia* (Fig. 1) and recording its effects.

Fig. 1. Gel showing the PCR products of *16S rRNA* primer that amplifies around 900 bp of *Wolbachia* in the following uzifly populations. Lane 1. Mass ruler; Lane 2 and 3 control populations from field and laboratory populations; Lane 4 tetracycline treated uzifly population; Lane 5 uziflies emerged from tetracycline treated host silkworms; Lane 6 duplicate laboratory population; Lane -7 temperature treated population; Lane 8 and 9 two and three week old uzifly populations. Arrow shows 900 bp.



The study revealed that the fecundity of infected females were greater by around 17% than that of uninfected females irrespective of the presence of *Wolbachia* in males and yielded 552.192 ± 2.3 (n = 26) and 550.8 ± 2.84 (n = 25) in the crosses of infected males x infected females and uninfected males x infected females respectively, when compared to uninfected females which yielded a lower fecundity of 459.42 ± 4.605 (n = 24) and 432.46 ± 3.79 (n = 24) (f = 4.108, d. f. = 98, P < 0.01) in the crosses of uninfected males x uninfected females and in infected males x uninfected females respectively, assuming a 99% confidence limit (Fig. 2).

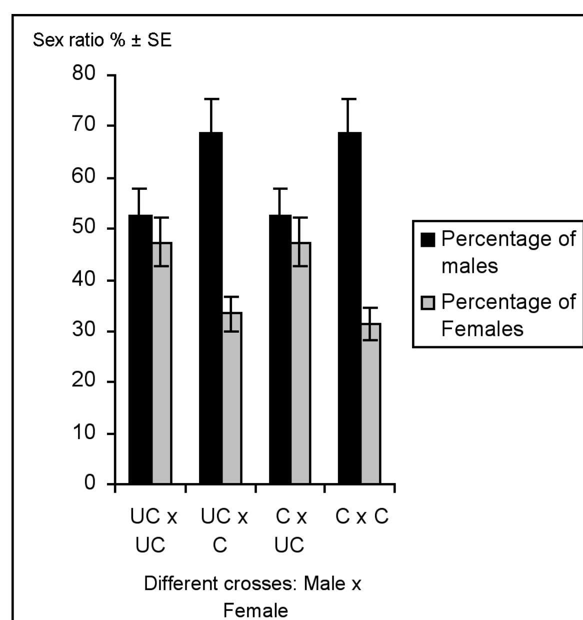
Fig. 2. Fecundity and Hatchability in cured and uncured populations. C = Cured population (uninfected); UC = Uncured population (infected).



The uzifly on the other hand displays, lower levels of sterility accounting for from 64 % to 70% of embryonic development in crosses between both sexes from the infected population and in the crosses of uninfected males with infected females yielding 388.77 ± 2.14 (n = 26) and 351 ± 3.15 (n = 25) respectively. However, it was reduced to 33 - 35% of embryonic development in the crosses of both males and females from uninfected populations and yielded 151.75 ± 3.78 (n = 24). The cytoplasmic incompatibility was displayed in the crosses of infected males with uninfected females as 10 - 13 % of embryonic development which yielded 59.04 ± 1.95 (n = 24) (f= 13.4, d. f. = 98. P < 0.01) (the values are mean hatchability number ± SE) assuming a 99% confidence limit (Fig. 2).

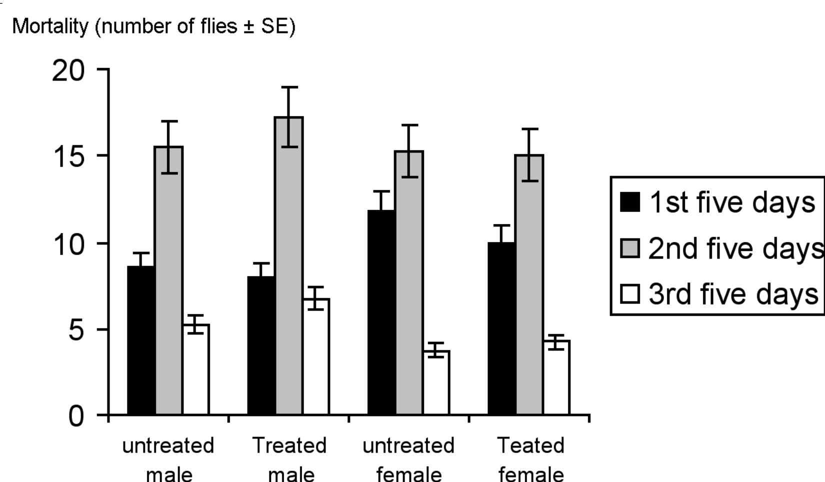
The sex ratio revealed variation in infected and uninfected females. The percentages of males in the crosses of infected males x infected females was 52.65 ± 0.14% (n = 26) and in the uninfected males x infected females was 52.614 ± 0.17% (n = 25), whereas in the crosses of uninfected males x uninfected females was 68.59 ± 0.4% (n = 24) and in the infected males x uninfected females was 68.66 ± 0.464% (n = 24) (f = 270.017, d. f. = 98, P < 0.01) assuming a 99% confidence limit (Fig. 3).

Fig. 3. Sex ratio in crosses of cured and uncured populations. UC= Uncured population (infected population); C = Cured population (uninfected population).



The results also revealed that the tetracycline did not affect longevity of uziflies. Among thirty-five flies in each replicate, the males in both control and tetracycline administered uziflies died in varied number of days within seventeen days. There was no significant variation in the life span of the male populations with 8.5 ±0.66, 15.5 ± 1.59 and 5.25 ± 0.75 (n = 5) in the control compared with 8 ± 0.35, 17.25 ± 0.8 and 6.75 ± 2.25 (n = 5) in treated replicates (t = 0.33, d. f. = 8, P > 0.05; t = -0.49, d. f. = 8, P > 0.05 and t = -1.14, d. f. = 8, P > 0.05) during the first five days, second five days and third five days, respectively. The life span of female flies in the first five days in control batches showed significantly increased mortality when compared to tetracycline administered batches and yielded 11.75 ± 0.94 and 10 ± 0.89 (n = 5) respectively (t = 3.88, d. f. = 8, P < 0.05). However, in the second five days and the third five days there was no significant variation in mortality rates which yielded 15.25 ±

Fig. 4. Mortality of uziflies in tetracycline treated and control batches.



0.77; 15 ± 0.46 and 3.75 ± 0.37; 4. 25 ± 0.31 ($t = 0.19$, d. f. = 8, $P > 0.05$ and $t = 0.72$, d. f. = 8, $P > 0.05$), respectively (Fig. 4).

Discussion

In the present study, we have estimated the effects of *Wolbachia* infection on the reproductive fitness of the uzifly. The uzifly harbours high prevalence of *Wolbachia* in both field and laboratory populations. The higher rates of fecundity and egg hatch in *Wolbachia* -infected females compared to uninfected females indicate that *Wolbachia* may be essential for host development, survival and reproduction to boost their own reproductive success. Similar observations have been made in the wasp *Asobara tabida* where it was shown that the reduced fecundity is associated with the absence of a particular strain of *Wolbachia* (Dedeine et al., 2004). They further ruled out the possibility that the inhibition of egg production is caused directly by antibiotics or indirectly through the release of endotoxin from decaying bacteria (Dedeine et al., 2001). These results further supports our data that egg production depends on the presence of *Wolbachia*.

Further, *Wolbachia* is likely the causative agent of cytoplasmic incompatibility in crosses between uninfected females with infected males of *E. sorbillans* that resulted in 88-90 % embryonic mortality. In reciprocal crosses, the compatibility was restored. In agreement with Bourtzis and O'Neill (1998) it can be suggested that in infected females, the *Wolbachia* in the egg can rescue the effects of the same strain of *Wolbachia* in developing sperm which is due to successful completion of karyogamy and subsequent normal development of the embryos. Whereas, uninfected females which cannot rescue the sperm modification show developmental lags in offspring production. Cytoplasmic incompatibility caused by *Wolbachia* is of considerable interest for the biological control of pest arthropods (Caspari et al., 1959). Early studies have focused on methods for eradication of host populations analogous to those used for the sterile male technique (Laven, 1967).

Thirty to 36% egg mortality was recorded in the infected female flies irrespective of the presence of *Wolbachia* in males, but 64 to 70% egg mortality occurred in the crosses within the uninfected population. This suggests that *Wolbachia* may be essential for normal reproduction of the uzifly. This might be due either to uninfected females laying a large proportion of unfertilised eggs, or decreased oocyte production in the uninfected females. Further, uninfected males had no effect on offspring production.

The sex ratio of offspring from crosses of infected males with infected females and uninfected males with infected females were approximately 1:1, however there was a slight deviation from this ratio with increased 2 to 4% males. In the crosses between infected males x uninfected females and uninfected males x uninfected females, the sex ratio was approximately 2:1 male: female among surviving offspring. It is possible that the male-biased sex ratio in uninfected females was due

to the death of female offspring as has explained by the mechanism that the *Wolbachia* increased its vertical transmission via killing uninfected females. Further, it was also found that tetracycline did not affect longevity of uziflies. Therefore, it can be inferred that reproduction in the uzifly, *Exorista sorbillans*, may be considered as being dependent on the presence of *Wolbachia*.

Acknowledgements

The authors are thankful to DBT-New Delhi, India for financial assistance (Vide ref. No. BT/PR2439/PBD/10/06/2001) and to Dr. John Milne, Mahidol University, Bangkok, for reading the manuscript.

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